Glycerin Monostearate Aggravates Male Reproductive Toxicity Caused by Di(2-ethylhexyl) Phthalate in Rats*

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Summary: Human beings are increasingly exposed to phthalates, which are a group of chemicals used to make plastics more flexible and harder to break, and simultaneously ingesting abundant food emulsifiers via daily diet. The purpose of this study was to investigate the effect of the food emulsifier glycerin monostearate (GMS) on male reproductive toxicity caused by di(2-ethylhexyl) phthalate (DEHP, one of the phthalates) and explore the underlying mechanism. Thirty male Sprague-Dawley rats were randomly divided into control group, DEHP group and DEHP+GMS group. Rats in the DEHP group and DEHP+GMS group were orally administered with 200 mg/kg/d DEHP with or without 20 mg/kg/d GMS. After 30 days of continuous intervention, it was found that the serum testosterone level was significantly lowered in DEHP group and DEHP+GMS group than that in control group (P<0.01). The serum testosterone level and the relative testis weight were significantly decreased in the DEHP+GMS group as compared with those in the DEHP group and control group (P<0.05). More spermatids were observed to be shed off in DEHP+GMS group than in DEHP group. The expression levels of cell cycle checkpoint kinase 1 (Chk1), cell division cycle gene 2 (Cdc2), and cyclin-dependent kinase 2 (CDK2) were down-regulated in DEHP group, and this tendency was more significant in DEHP+GMS group (P<0.05 or P<0.01). There was no significant difference in the P-glycoprotein (P-gp) expression between DEHP group and control group. However, P-gp was markedly down-regulated in DEHP+GMS group (P<0.01). The results indicated that the food emulsifier GMS aggravated the toxicity of DEHP on male reproduction by inhibiting the cell cycle of testicular cells and the expression of P-gp in testis tissues.
Key words: glycerin monostearate; di(2-ethylhexyl) phthalate; reproductive toxicity; cell cycle; P-glycoprotein

Phthalates (PEs), as a kind of plasticizer, can increase the flexibility of the plastic and facilitate its processing. They are widely found in various plastic products, even with high concentrations. According to the China National Food Safety Standard GB9685-2016, the concentrations of some PEs in food contact plastic materials and products could be up to 43%. The PEs and plastic polymer materials are weakly combined, with hydrogen bond, van der Waals force, etc. Therefore, PEs can easily escape from plastic products, and enter the environment. Nowadays, human beings are increasingly exposed to ubiquitous PEs throughout their whole lifetime via diet, breathing and skin. As reported, the total PEs exposure level in China is 23–159 μg/kg/d[1]. Di(2-ethyhexyl) phthalate (DEHP) is the major component of PEs and its exposure level is 11–116 μg/kg/d, which is at the critical level closed to the value of DEHP's tolerable daily intake (TDI, 20–140 μg/kg/d)[1]. Previous studies have shown that DEHP exposure could cause damage to reproductive function in animals, and the DEHP level causing no observed adverse effect was 5 mg/kg/d[2, 3]. Studies suggested that DEHP exposure could lead to decrease of sperm concentration and motility, increase of deformity rate, and induce asthenospermia[4, 5].

Dietary exposure is the major pathway of people exposed to PEs, such as DEHP[6]. However, their absorption, transport and metabolism in the gastrointestinal tract are affected by various factors such as nutritional status of the body, food matrix, and the physical property of contaminants[7]. In food

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processing, food additives, such as emulsifiers, can increase the permeability of intestinal cells[8]. Therefore, food emulsifiers are also commonly used in the pharmaceutical industry to promote the bioavailability of difficult-to-absorb drugs[8]. Our previous studies have confirmed that the food emulsifier Tween 80 (25 mg/kg, intragastrical administration, ig) and glycerin monostearate (GMS, 200 mg/kg, ig) could promote the bioavailability of PEs and increase their internal exposure levels, thereby increasing their toxicological effects[7, 9, 10].

There are multiple barriers blocking the entry of contaminants in the body. For example, P-glycoprotein (P-gp) efflux system, encoded by the multidrug resistance gene *MDR1*, is widely distributed in intestinal epithelial cells, blood-testis barrier, blood-brain barrier and other tissues, which can hinder the entry of toxic and harmful substances into cells. P-gp can also pump exogenous macromolecule out of the cells[10, 11]. Our previous work indicated that Tween 80 could damage the structure and function of mitochondria in enterocytes, decrease the respiration rate, and then inhibit the function of P-gp ATPase activity, thus impairing the barrier function of P-gp, and increase the absorption of DEHP[10]. It was reported that inhibition of P-gp expression (or activity) could not only reverse drug resistance, but also improve cell membrane permeability[11]. Therefore, we hypothesized that the effect of emulsifiers on the toxic effects of PEs may be related to the altered expression levels of P-gp.

Increased exposure to DEHP and GMS may increase the reproductive safety risk in males[1]. Based on our previous studies[7, 12], the present study aimed to explore the effect of GMS on the male reproductive toxicity induced by DEHP and the underlying mechanism. Male rats were exposed to DEHP at 200 mg/kg/d with or without GMS (20 mg/kg/d) for 30 days. The testis weight, testosterone level, and histopathology were detected for the evaluation of toxicity on male reproduction. P-gp and cell cycle-related protein, including cell cycle checkpoint kinase 1 (Chk1), cell division cycle gene 2 (Cdc2) and cyclin-dependent kinase 2 (CDK2) were measured to explore the underlying mechanism.

1 MATERIALS AND METHODS

1.1 Reagents and Instruments

5430R high-speed refrigerated centrifuge was provided by Eppendorf (Hamburger, Germany), IKA T10 high speed disperser by IKA (Staufen, Germany), and Thermo Scientific microplate reader by Thermo Fisher Scientific (Waltham, USA). DEHP (CAS 117-81-7, purity 99%) was purchased from Sinopharm (Shanghai, China). GMS (food grade) and commercial assay kits of testosterone for rats were gained from Nanjing JianCheng Bioengineering Institute (Nanjing, China). Secondary antibodies, BCA Protein Assay Kit and RIPA cell lysis buffer were purchased from Beyotime (Haimen, China), and primary antibodies against P-gp, β-actin, CDK2, Cdc2 and Chk1 were from Abcam (Cambridge, USA).

1.2 Animals

Male Sprague-Dawley (SD) rats (SPF grades, 1 month, weighing about 200 g) were obtained from Shanghai Jiesijie Experimental Animal Co., Ltd., [License No.: SCXK (HU) 2013-0006].

1.3 Experimental Design

Thirty male SD rats were randomly divided into control group, DEHP group and DEHP+GMS group, with 10 rats in each group. According to the previous studies[7, 8], rats in the DEHP group and DEHP+GMS group were orally administered with 200 mg/kg/d DEHP in 0.5% sodium carboxymethylcellulose (CMC-Na) with or without 20 mg/kg/d GMS. Rats in the control group were orally administered with isodose 0.5% CMC-Na. The intervention lasted for 30 days. At the end of the experiment, about 1.0 mL blood of each anesthetized rat was collected from the caudal vein by cutting off the tip of their tails. The serum was prepared with centrifugation at 3000 r/min for 10 min. Serum testosterone level was measured using the commercial assay kit of testosterone. All rats were fasted overnight (12 h) and euthanized using 4% Fluothane in oxygen in an airtight container. The body and the testes of each rat were weighed. Relative weight of testis was calculated by the formula: Relative testis weight=Testis weight (g)/Body weight (g)×100%. The whole left testes were fixed in 10% formalin solution for hematoxylin-eosin (HE) staining. The serum and the right testes were stored at –80°C for further analyses.

Animal experiments were conducted following the Declaration of Helsinki (as revised in Edinburgh 2000) and the local regulations. Furthermore, all experiments were approved and supervised by the Animal Care and Use Committee and the Animal Ethics Committee at Wenzhou Medical University.

1.4 Histopathological Observation of Testis

The left testis tissues were fixed in 10% buffered formalin in PBS (pH 7.4), embedded in paraffin, sliced in 4 μm thick sections and stained with HE. Morphological analysis was performed by a senior pathologist who was blinded to the experimental design. HE-stained samples were observed under a TS100 inverted microscope (Nikon, Japan), and photographed under ×200 magnification.

1.5 Western Blotting

Homogenized testis tissues (50 mg) in pre-cold 1600 μL radioimmunoprecipitation assay (RIPA) lysis buffer containing 320 μL phenylmethanesulfonyl fluoride (PMSF) were centrifuged at 14 000 r/min for 30 min. The supernatants were used for the

determination of protein concentrations using the BCA Protein Assay Kit. After the denaturation at 100°C for 10 min, the protein samples (in 5× loading buffer) were subjected to 11% SDS-polyacrylamide gel electrophoresis, and then transferred to polyvinylidene fluoride (PVDF) membrane. After the block in Tris-buffered saline and Tween 20 (TBST) buffer containing 5% non-fat dry milk overnight at 4°C, and followed by washing, 1:2000–1:1000 diluted primary antibodies for the interesting targets were respectively added for incubation overnight at 4°C. Then the corresponding 1:1000 diluted secondary antibodies were added for 3-h incubation at room temperature. β-actin was used as internal control. The samples were visualized using 3,3'-diaminobenzidine (DAB) detection system.

1.6 Statistical Methods

All statistical analyses were performed using Statistical Product and Service Software, version 17.0 (SPSS Inc., USA). Values are expressed as means and their standard deviations (SD). One-way ANOVA was used to compare the difference between groups. The least significant different *t* test was used for homogeneity of variance. Dunnett's *T* test was used for multiple comparisons. $P<0.05$ was considered statistically significant. All *P* values were two tailed.

2 RESULTS

2.1 Body Weight and Testis Weight of Rats

As shown in fig. 1, the body weight and testis weight were slightly decreased in DEHP group and DEHP+GMS group as compared with the control group, with no significant differences noted. The relative testis weight was significantly reduced in DEHP+GMS group when compared with that in the control group ($P<0.05$).

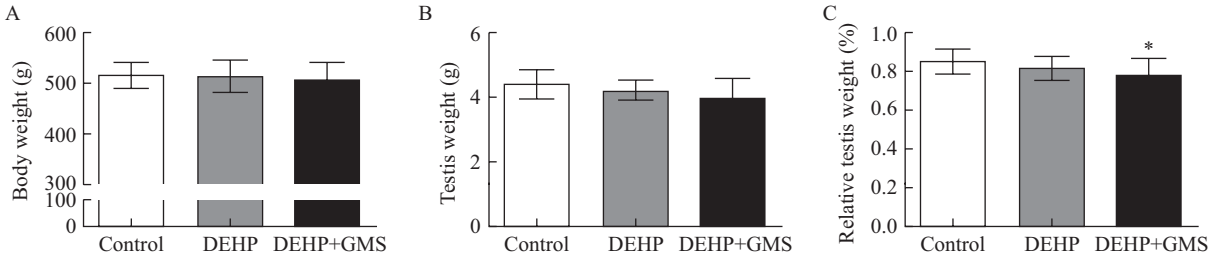

Fig. 1 Body weight and testis weight
A: bodyweight; B: testis weight; C: relative weight of testis. $^{*}P<0.05$ *vs.* control group

2.2 Testosterone Levels in Rats

The serum testosterone levels were significantly lower in the DEHP group and DEHP+GMS group than those in the control group, with the DEHP+GMS group having the lowest level of serum testosterone ($P<0.05$ or $P<0.01$, fig. 2).

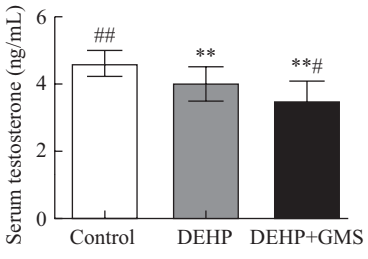

Fig. 2 Serum testosterone level of rats
$^{**}P<0.01$ *vs.* control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs.* DEHP group

2.3 Histopathological Observation of Rat Testes

HE staining showed that the spermatids were in the order of spermatogonium, primary spermatocyte and spermatid from the basal lamina of the seminiferous tubules with distinguishable layers in control group (fig. 3A). However, deciduous spermatids were observed in DEHP group (fig. 3B) and they were significantly increased in DEHP+GMS group (fig. 3C), indicating that GMS aggravated the toxicity of DEHP on testicular tissues.

2.4 Expression of Cell Cycle-related Proteins in Testes

The expression levels of Chk1 and Cdc2 were significantly down-regulated in DEHP group and DEHP+GMS group as compared with the control group ($P<0.05$ or $P<0.01$), with their levels being the lowest in DEHP+GMS group ($P<0.05$ or $P<0.01$, fig. 4).

2.5 P-gp Expression Levels in Testicular Tissue Cells

As shown in fig. 5, the P-gp expression level in DEHP+GMS group was significantly lower than that in the control group and DEHP group ($P<0.01$). However, no significant difference was observed between control group and DEHP group ($P>0.05$).

3 DISCUSSION

Testosterone is mainly excreted by ovaries in female and testes in male, and minimally by the adrenal glands. Combined with androgen-binding protein, testosterone can maintain a high concentration in the seminiferous tubules, which could promote spermatogenesis, maintain male secondary sexual

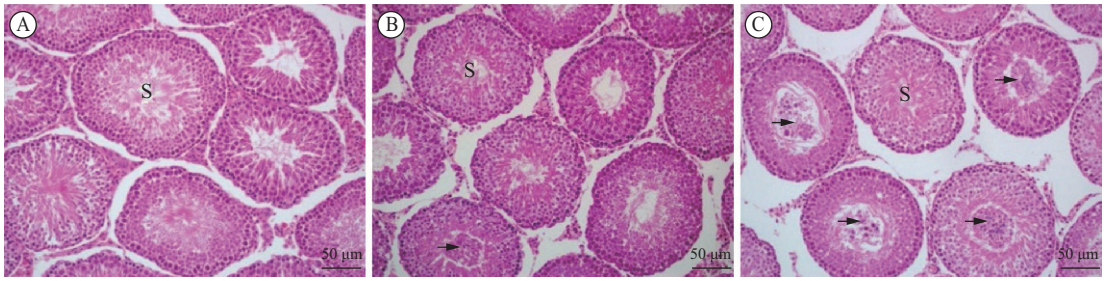

Fig. 3 Pathological morphology of testis tissues (HE staining, ×200)
A: control group; B: DEHP group; C: DEHP+GMS group. S: seminiferous tubule; →: deciduous spermatids

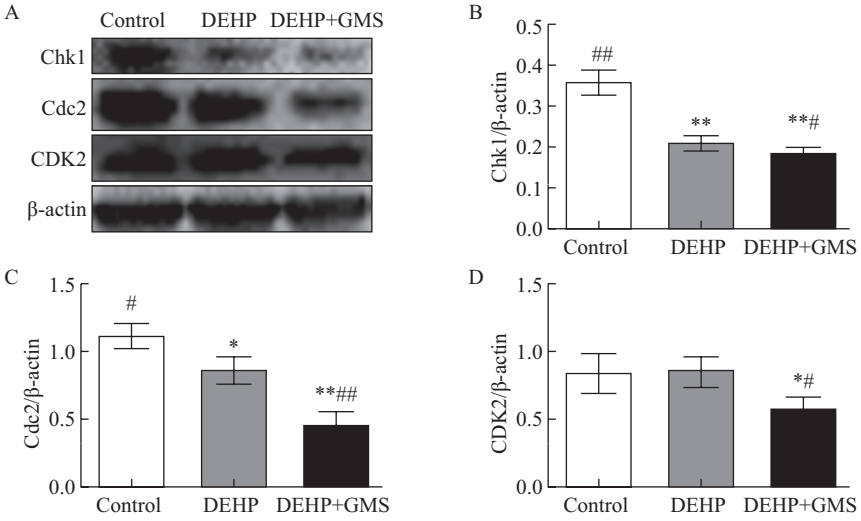

Fig. 4 Western blot analysis of the expression of cell cycle-related proteins Chk1, Cdc2 and CDK2 in rat testis
The relative expression of each cell cycle protein was obtained by normalization against β-actin (an inner-control). Data are presented as mean±SD (n=3; $^{*}P$<0.05, $^{**}P$<0.01 *vs.* control group; $^{\#}P$<0.05, $^{\#\#}P$<0.01 *vs.* DEHP group).

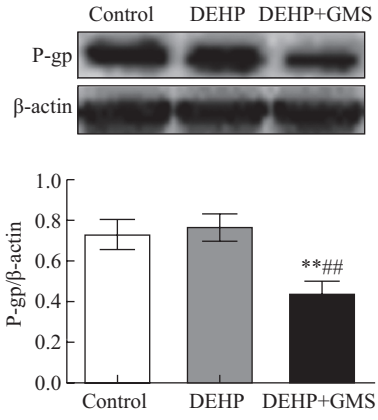

Fig. 5 Expression of P-gp in rat testis by Western blotting
The relative expression of P-gp was obtained by normalization against β-actin (an inner-control). Data are presented as mean±SD (n=3; $^{**}P$<0.01 *vs.* control group; $^{\#\#}P$ <0.01 *vs.* DEHP group).

characteristics and physiological functions. Therefore, alterations of testosterone level indicate the male reproductive toxicity. PEs exposure can cause reproductive toxicity, and testis is the major target organ[13]. Studies have shown that DEHP exposure during pregnancy could lead to testicular dysfunction, genital malformations, etc. in male offsprings[14]. Noriega *et al*[15] argued that DEHP (300 and 900 mg/kg/d) exposure reduced serum testosterone levels in SD rats and LE rats. Akingbemi *et al*[16] reported that DEHP (10 and 100 mg/kg/d) could promote interstitial cells hyperplasia and inhibit testosterone synthesis, but increased the levels of serum testosterone and luteinizing hormone (LH) in rats. In our study, the serum testosterone level was decreased significantly in DEHP group, and the decrease was more profound in the presence of food emulsifier GMS. These results indicated that GMS might aggravate the reproductive toxicity of DEHP. The decrease of relative testis weight and much more deciduous spermatids further added the evidence to this indication.

In the injured testis, disordered cell cycle progression may occur in the spermatids, which is controlled by several key proteins, such as Chk1, Cdc2 and CDK2. In the current study, Western blotting showed that the expression levels of Chk1, Cdc2 and CDK2 were significantly down-regulated in the rats

exposed to DEHP. Chk1 is an important kinase in cell cycle, and its inhibition will cause cell arrest and apoptosis[17]. Cdc2 could control the initiation of the cell cycle and the transition of each phase. Its mutation could cause cell-cycle arresting at G2/M phase[18]. CDK2 and cyclin E could form a complex, which promotes G1-S transition. The inhibition of CDK2 will cause cell-cycle arresting at G1/S phase[19, 20]. Lin *et al*[21] reported that gestational DEHP exposure could inhibit the expression of Cdc2 in the brain of neonatal rats and impede the normal development of brain cells. As reported, DEHP could also induce apoptosis by up-regulating p53 and Bax, or by down-regulating Bcl-2[22, 23]. The results of this study indicated that DEHP might cause cell cycle arrest of testicular cells, and induce apoptosis, and result in the shedding of spermatids in the testicular seminiferous tubule.

The decrease of relative testis weight and serum testosterone levels, and the pathological morphological changes of testes in our study suggested that GMS might aggravate DEHP's male reproductive toxicity, which, we speculate, might be related to the increased bioavailability of DEHP. Our preliminary work showed that emulsifiers could improve the bioavailability of PEs[9, 10]. GMS was reported to improve the bioavailability of PEs, and the relative bioavailability of six PEs was increased by 1.30–1.48 times[7]. As aforementioned, there are multiple barriers in the body that can block the entry of contaminants. For example, P-gp could hinder the entry of toxics and harmful substances into cells. On the other hand, P-gp could also pump exogenous macromolecules out from cells[10, 11]. An *in vitro* study showed that food emulsifier Tween 80 could injury the barrier function of P-gp by inhibiting the respiration of mitochondria and hindering the energy supply[10]. Barta *et al*[24] reported that GMS could inhibit the activity and expression of P-gp in Caco-2 cell membrane. This study showed that P-gp was down-regulated in testis tissues exposed to GMS, which might inhibit the barrier function of P-gp and thus result in the increase of the internal exposure of DEHP, and aggravation of male reproductive toxicity in rats.

In addition, synergistic effect of the toxicity was found among various PEs, which might exacerbate the toxicity of PEs[25]. Food emulsifiers are widely used in processed foods. Epidemiological studies showed that the autoimmune disease incidence is positively correlated with the consumption of emulsifiers[11, 26, 27]. Our study demonstrated that DEHP exposure caused male reproductive toxicity by inhibiting the cell cycle of testicular cells; food emulsifier GMS exacerbated male reproductive toxicity caused by DEHP, which might result from the down-regulated expression of P-gp.

Diet is the major pathway through which human beings are exposed to contaminants, which are usually chronic, continuous and cumulative. Food emulsifiers could promote the absorption of dietary contaminants and increase their exposure levels, which might lead to the increase of food safety risks. We believe that it is necessary to improve the related management measures to lower food safety risks caused by food emulsifiers.

Conflict of Interest Statement

The authors report no conflicts of interest in this study.

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